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[Title of Invention] ORGANIC ANION TRANSPORTER AND GENE CODING FOR THE SAME

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[List of Document(s)]

[Name of Document]	Specification	1
[Name of Document]	Drawings	1
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[Title of the Invention] Organic Anion Transporter and Gene Thereof

[Claims]

[Claim 1] Protein selected from the following (A), (B), (C) and (D).

(A) Protein comprising an amino acid sequence represented by SEQ ID No. 1;

(B) Protein comprising an amino acid sequence where one or several amino acid(s) is/are deleted from, substituted for or added to the amino acid sequence represented by SEQ ID No. 1 and having a capability of transporting an organic anion;

(C) Protein comprising an amino acid sequence represented by SEQ ID No. 2; and

(D) Protein comprising an amino acid sequence where one or several amino acid(s) is/are deleted from, substituted for or added to the amino acid sequence represented by SEQ ID No. 2 and having a capability of transporting an organic anion.

[Claim 2] The protein according to claim 1, wherein it is derived from human being.

[Claim 3] The protein according to claim 1, wherein it is derived from rat.

[Claim 4] The protein according to claim 1, wherein it is derived from renal tissues.

[Claim 5] Gene coding for the protein mentioned in

claim 1.

[Claim 6] Gene comprising DNA selected from the following (a), (b), (c) and (d).

(a) DNA comprising a base sequence represented by SEQ ID No. 1;

(b) DNA hybridizing to the DNA comprising the base sequence represented by SEQ ID No. 1 under a stringent condition and coding for protein which has a capability of transport of an organic anion;

(c) DNA comprising a base sequence represented by SEQ ID No. 2;

(d) DNA hybridizing to the DNA comprising the base sequence represented by SEQ ID No. 2 under a stringent condition and coding for protein which has a capability of transport of an organic anion.

[Claim 7] The DNA according to claim 6, wherein it is derived from human being.

[Claim 8] The Gene according to claim 6, wherein it is derived from rat.

[Claim 9] The Gene according to claim 6, wherein it is derived from renal tissues.

[Claim 10] Plasmid containing a region coding for the gene mentioned in any of claims 5 to 9 or protein in the said gene.

[Claim 11] The plasmid according to claim 10, wherein

it is an expression plasmid.

[Claim 12] A host cell which is transformed by the plasmid mentioned in claim 10.

[Claim 13] Nucleotide containing a partial sequence of continuous 14 or more bases in the base sequence represented by SEQ ID No. 1 or No. 2 or a complementary sequence thereof.

[Claim 14] The nucleotide according to claim 13, wherein it is used as a probe for the detection of gene which codes for a protein having a capability of transport of an organic anion.

[Claim 15] The nucleotide according to claim 13, wherein it is used for modulation of expression of gene which codes for a protein having a capability of transport of an organic anion.

[Claim 16] Antibody to the protein mentioned in any of claims 1 to 4.

[Claim 17] A method of using the protein mentioned in any of claims 1 to 4 for the test of action of a test substance as a substrate for the capability of the said protein to transport an organic anion.

[Detailed Description of the Invention]

[0001]

[Technical Field to which the Invention Belongs]

The present invention relates to a gene participating in transport of an organic anion and also to a polypeptide for

which the gene codes.

[0002]

[Prior Art]

Kidney plays an important role for excretion of xenobiotics and drugs outside the body. Anionic drugs are excreted into urine from urinary tubule near the kidney by a route mediated by a carrier. Excretion of an organic anion as such starts by the fact that the urinary tubule cells take up the organic anion from the blood near the urinary tubule via the basolateral membrane.

[0003]

With regard to the uptake of an organic anion by the basolateral membrane, investigations have been carried out by an experiment by means of a perfusion of excised organ or an isolated cell membrane vehicle system using, for example, a p-aminobenzoate as an organic anion of the substrate. During the investigations, it has been believed that an organic anion transporter is participated in the uptake of an organic anion and that the uptake of an organic anion in a basolateral membrane is mediated by an exchange transporter for organic anion with dicarboxylic acid.

[0004]

However, in the conventional means, it is difficult to analyze the details of the transport mechanism in urinary tubules such as a network of transport among transporters and an

interaction among drugs during the process of renal excretion and there has been a demand that gene of an organic anion transporter is isolated so as to make the detailed function analysis possible.

[0005]

With regard to an organic anion transporter gene expressed in liver, various molecular species have been cloned (Hagenbuch, et al., *Proc. Natl. Acad. Sci. USA*, volume 88, page 10629, 1991; Jacquemin, et al., *Proc. Natl. Acad. Sci. USA*, volume 91, page 133, 1994; Shi, et al., *J. Biol. Chem.*, volume 270, page 25591, 1995; and Kanai, et al., *Am. J. Physiol.*, volume 270, page F319, 1996). A genetic cloning of OCT1 which is one of the organic cation transporters expressed in kidney and liver has been also reported (Grundemann, et al., *Nature*, volume 372, page 549, 1994).

[0006]

As to a transporter for dicarboxylic acid, a genetic cloning of sodium-dependent dicarboxylate transporter (NaDC-1) in kidney has been also reported (Pajor, et al., *J. Biol. Chem.*, volume 270, page 5779, 1995).

[0007]

Recently, as an analogous gene of sodium-independent rat liver organic anion transporter (oatp), cloning of gene of an organic anion transporter OAT-K1 localized in renal urinary tubules of rat has been reported (Saito, et al., *J. Biol. Chem.*,

volume 270, page 20719, 1996). However, with regard to the OAT-K1, it has not been confirmed yet that its transport mechanism is due to an exchange transport between an organic anion and a dicarboxylic acid.

[0008]

[Problems that the Invention is to Solve]

An object of the present invention is to provide a novel organic anion transporter gene participating in an organic anion transport in the kidney and also to provide an organic anion transporter which is a polypeptide for which the gene codes. Other objects will be apparent from the following descriptions.

[0009]

[Means for Solving the Problems]

The present inventors have cloned the gene of a novel protein having a capability of transporting an organic anion from renal cells of rats and further cloned a human homologous gene (homolog). They have moreover succeeded in confirming the transporting ability of an organic anion by expressing the product of those genes in oocytes of *Xenopus* whereupon the present invention has been achieved.

[0010]

Thus, the present invention relates to a protein selected from the following (A), (B), (C) and (D).

(A) Protein comprising an amino acid sequence represented by SEQ ID No. 1;

(B) Protein comprising an amino acid sequence where one or several amino acid(s) is/are deleted from, substituted for or added to the amino acid sequence represented by SEQ ID No. 1 and having a capability of transporting an organic anion;

(C) Protein comprising an amino acid sequence represented by SEQ ID No. 2; and

(D) Protein comprising an amino acid sequence where one or several amino acid(s) is/are deleted from, substituted for or added to the amino acid sequence represented by SEQ ID No. 2 and having a capability of transporting an organic anion.

[0011]

The present invention further relates to a gene comprising DNA selected from the following (a), (b), (c) and (d)。

(a) DNA comprising a base sequence represented by SEQ ID No. 1;

(b) DNA hybridizing to the DNA comprising the base sequence represented by SEQ ID No. 1 under a stringent condition and coding for protein which has a capability of transport of an organic anion;

(c) DNA comprising a base sequence represented by SEQ ID No. 2;

(d) DNA hybridizing to the DNA comprising the base sequence represented by SEQ ID No. 2 under a stringent condition and coding for protein which has a capability of transport of an organic anion.

[0012]

In living bodies, the novel protein having a capability of transporting an organic anion according to the present invention or, in other words, an organic anion transporter (OAT1) is mostly expressed in urinary tubules of the kidney.

[0013]

Further, an organic anion transporting ability (uptake of an organic anion into expressed cells) of the organic anion transporter OAT1 is activated by the presence of a dicarboxylic acid. It is therefore likely to be a transporter which carries out an exchange transport between an organic anion and a dicarboxylic acid. Further, in the exchange transport, it is likely that the dicarboxylic acid which comes outside the cells in exchange for the organic anion by OAT1 is taken up into the cells by a sodium-dependent dicarboxylic acid transporter (NaDC-1) for recycling.

[0014]

Furthermore, the organic anion transporter OAT1 of the present invention has a substrate selectivity of a very broad range having a capability of transporting (taking up) various drugs having different structures such as cyclic bases, prostaglandins, uric acid, antibiotic substances, nonsteroidal anti-inflammatory agents, diuretics and anti-tumor agents.

[0015]

Still further, it is likely that the organic anion

transporter OAT1 of the present invention has no homology to the already-reported organic anion transporter OAT-K1 from kidney of rats but is an entirely different molecular species.

[0016]

[Mode for Carrying Out the Invention]

SEQ ID No. 1 in the Sequence Listing which will be mentioned later shows an amino acid sequence (551 amino acids) of protein encoded to the full-length cDNA base sequence (about 2.2 kbp) of gene of an organic anion transporter (rat OAT1) derived from kidney of rats and a translation region thereof.

[0017]

SEQ ID No. 2 shows an amino acid sequence (563 amino acids) of protein encoded to the full-length cDNA base sequence (about 2.2 kbp) of gene of an organic anion transporter (human OAT1) derived from kidney of human being and a translation region thereof.

[0018]

With regard to the base sequences or the amino acid sequences shown in the above SEQ ID Nos. 1 and 2, a homology search was carried out for all sequences included in the already-known DNA databases (GenBank and EMBL) and protein databases (NBRF and SWISS-PROT) and, as a result, there was no identical one therein whereupon those sequences are thought to be novel ones.

[0019]

With regard to the protein of the present invention, in addition to that which has an amino acid sequence represented by SEQ ID No.1 or 2, those in which one or more amino acid(s) in the amino acid sequence represented by SEQ ID No. 1 or 2 is/are deleted, substituted or added may be exemplified. Deletion, substitution or addition of amino acid(s) may be within such an extent that an organic anion transport activity is not lost and the number(s) is/are usually from 1 to about 110 or, preferably, from 1 to about 55. Such an amino acid has a homology in terms of an amino sequence to the amino acid sequence represented by SEQ ID No. 1 or 2 to an extent of usually 1-80% or, preferably, 1-90%.

[0020]

With regard to the gene of the present invention, there are exemplified that which contains DNA being able to hybridize to DNA having a base sequence represented by SEQ ID No. 1 or 2 under a stringent condition in addition to that containing DNA having a base sequence represented by SEQ ID No. 1 or 2. The DNA which is able to hybridize as such may be in such an extent that protein for which the DNA is coded has a capability of transporting the organic anion. Such a DNA has a homology of usually 70% or more or, preferably, 80% or more homology to the base sequence represented by SEQ ID No. 1 or 2. Such a DNA includes mutant gene which is found in nature, mutant gene which is artificially modified and homologous gene derived

from other living body.

[0021]

In the present invention, hybridization under a stringent condition is usually carried out in such a manner that hybridization is conducted in a hybridization solution of 5 × SSC or similar salt concentration thereto at the temperature condition of 37 to 42°C for about 12 hours, a preliminary washing is conducted if necessary with a solution of 5 × SSC or similar salt concentration thereto and then a washing is conducted with a solution of 1 × SSC or similar concentration thereto. In order to achieve a higher stringency, it can be done where a washing is conducted in a solution of 0.1 × SSC or similar salt concentration thereto.

[0022]

It is also possible that the organic anion transporter gene according to the present invention is isolated and obtained by means of a screening where tissues or cells of appropriate mammals are used as a gene source. With regard to the mammals, human being may be exemplified in addition to non-human ones such as dogs, cattle, horses, goats, sheep, monkeys, pigs, rabbits, rats and mice.

[0023]

Screening and isolation of the gene may be advantageously carried out by means of an expression cloning, etc.

[0024]

For example, renal tissues of rats are used as a gene source and mRNA (poly(A)$^+$RNA) is prepared therefrom. This is fractionated and each of the fractions is introduced into oocytes of *Xenopus* together with cRNA of rat sodium-dependent dicarboxylate transporter (NaDC-1).

[0025]

cDNA of NaDC-1 gene has been reported already (Pajor, et al., *J. Biol. Chem.*, volume 270, page 5779, 1995) and, therefore, it is possible to easily prepare the cDNA of NaDC-1 gene from the sequence information using a PCR or the like. Starting from the resulting NaDC-1 cDNA, it is possible to synthesize RNA (cRNA) (being capped) which is complementary thereto using, for example, T3 or T7 RNA polymerase.

[0026]

With regard to the oocytes into which mRNA and NaDC-1 cRNA are introduced, transport (uptake) of a substrate into the cells is measured using, for example, p-aminohippuric acid (PAH) as a substrate (organic anion) and the fraction of mRNA showing a high uptake is selected whereupon mRNA in OAT1 can be concentrated. A cDNA library is prepared on the basis of the mRNA concentrated as such. cRNA (capped one) is prepared from cDNA of the library, each clone is introduced into oocytes together with NaDC-1 cRNA as same as before and positive clone is selected using the uptake activity of the substrate as an index whereupon it is possible to obtain clone containing cDNA

of the OAT1 gene.

[0027]

As to the resulting cDNA, its base sequence is determined by a conventional method and a translated region is analyzed whereby the protein coded therefor or the amino acid sequence of OAT1 can be determined.

[0028]

The fact that the resulting cDNA is a cDNA of an organic anion transporter gene or, in other words, the gene product coded for cDNA is an organic anion transporter can, for example, be inspected as follows. Thus, cRNA prepared from the resulting OAT1 cDNA is introduced into oocytes to express and an ability of transporting (taking up) the organic anion into the cells can be confirmed as above by measuring the uptake of the substrate into the cells by means of a conventional uptake experiment (Kanai and Hediger, *Nature*, volume 360, pages 467-471, 1992) using an appropriate organic anion as a substrate.

[0029]

It is also possible that the same uptake experiment is applied for the expression cells to investigate the characteristics of OAT1 such as a characteristic that OAT1 conducts an exchange transport with dicarboxylic acid and a substrate specificity of OAT1.

[0030]

When an appropriate cDNA library or genomic DNA library

prepared from a different genetic source is screened using cDNA of the resulting OAT1 gene, it is possible to isolate homologous gene, chromosomal gene, etc. from different tissues and different living bodies.

[0031]

It is further possible to isolate the gene from a cDNA library or a genomic DNA library by a conventional PCR (Polymerase Chain Reaction) using a synthetic primer designed on the basis of the information of the disclosed base sequence of the gene of the present invention (base sequence as shown in SEQ ID No. 1 or 2 or a partial sequence thereof).

[0032]

The DNA library such as a cDNA library and a genomic DNA library can be prepared by a method mentioned, for example, in "Molecular Cloning" (by Sambrook, J., Fritsch, E. F. and Maniatis, T.; published by Cold Spring Harbor Laboratory Press in 1989). Alternatively, when a commercially available library is available, that may be used as well.

[0033]

The organic anion transporter (OAT1) of the present invention may be produced, for example, by means of a gene recombination technique using cDNA coding for the organic anion transporter. For example, DNA (cDNA or the like) coding for the organic anion transporter is incorporated into an appropriate expression vector and the resulting recombinant

DNA can be introduced into an appropriate host cell. Examples of the expression system (a host-vector system) for the production of a polypeptide are expression systems of bacteria, yeasts, insect cells and mammalian cells. In order to obtain a functional protein among them, it is desirable to use insect cells and mammalian cells.

[0034]

For example, in order to express a polypeptide in mammals, DNA coding for an organic anion transporter is inserted into a downstream to an appropriate promoter (such as SV40, LTR promoter and elongation 1α promoter) in an appropriate expression vector (such as vector of a retrovirus type, papilloma virus vector, vaccinia virus vector and vector of an SV40 type) whereupon an expression vector is constructed. Then appropriate cells are subjected to a transformation using the resulting expression vector and the transformant is incubated in an appropriate medium to give a desired polypeptide. Examples of the mammal cells used as a host cell are cell strains including simian COS-7 cells, Chinese hamster CHO cells, human HeLa cells, primary culture cells derived from kidney tissues, LLC-PK1 cells derived from kidney of pig, OK cells derived from opossum kidney, etc.

[0035]

With regard to DNA which codes for an organic anion transporter OAT1, the cDNA having a base sequence shown in SEQ

ID No. 1 or 2 may be used for example and, in addition, it is not limited to the above-mentioned cDNA but DNA corresponding to the amino acid sequence is designed and the DNA coding for the polypeptide may be used. In that case, 1 to 6 kind(s) of codon has/have been known for coding for each amino acid and, although any codon may be selected for use, a sequence having higher expression can be designed when, for example, frequency of use of codon by the host utilized in the expression is taken into consideration. DNA having a designed base sequence can be obtained by chemical synthesis of DNA, fragmentation and bonding of the above-mentioned cDNA, a partial modification of the base sequence, etc. Artificial partial modification of and introduction of variation into a base sequence can be carried out by a site-specific mutagenesis (Mark, D. F., et al., *Proceedings of National Academy of Sciences*, volume 81, pages 5662 to 5666 (1984)), etc. utilizing a primer comprising a synthetic oligonucleotide coding for the desired modification.

[0036]

The nucleotide (oligonucleotide or polynucleotide) which hybridizes to the organic anion transporter gene of the present invention under a stringent condition can be used as a probe for detecting the organic anion transporter gene and, moreover, it can be used as antisense oligonucleotide, ribozyme, decoy, etc. for modulation of expression of the organic anion

transporter. With regard to such a nucleotide, a nucleotide containing a partial sequence of usually not less than continuous 14 bases in the base sequence represented by SEQ ID No. 1 or 2 or a complementary sequence thereof may be used for example and, in order to hybridize more specifically, longer sequence such as not less than 20 bases or not less than 30 bases may be used as a partial sequence.

[0037]

It is also possible that the organic anion transporter of the present invention or a polypeptide having the immunological homology thereto is used to obtain an antibody thereto and the antibody is able to be utilized for detection, purification, etc. of the organic anion transporter. The antibody can be manufactured using the organic anion transporter of the present invention or a fragment thereof or a synthetic peptide having a partial sequence thereof as an antigen. Polyclonal antibody can be manufactured by a conventional method in which an antigen is inoculated to a host animal (such as rat and rabbit) and immune serum is recovered while monoclonal antibody can be manufactured by a conventional way such as a hybridoma method.

[0038]

The present invention will now be further illustrated by way of the following Examples although those Examples do not limit the present invention.

[0039]

In the following Examples, each operation is carried out, unless otherwise mentioned, by a method mentioned in "Molecular Cloning" (by Sambrook, J., Fritsch, E. F. and Maniatis, T.; published by Cold Spring Harbor Laboratory Press in 1989) or according to the direction for use of the commercially available product when a commercially available reagent or kit is used.

[0040]

[Examples]

Example 1. Cloning of rat organic anion transporter

(1) Isolation of rat dicarboxylate transporter cDNA and preparation of cRNA

cDNA library was prepared from rat poly(A)$^+$ RNA using a kit for the synthesis of cDNA (trade name: SuperScript Choice System; manufactured by Gibco) and was integrated into a site of phage vector λ Ziplox (manufactured by Gibco) cleaved by a restriction enzyme EcoRI. Segment corresponding to the bases (from the 1323rd to the 1763rd) of rabbit sodium-dependent dicarboxylic acid transporter NaDC-1 gene (Pajor, et al., *J. Biol. Chem.*, volume 270, page 5779, 1995) was labeled with ^{32}P-dCTP and, using it as a probe, a cDNA library of rat was screened by PCR method. Hybridization was carried out for one night in a solution for hybridization of 37°C and the filter membrane was washed at 37°C with 0.1 × SSC/0.1% SDS. As to the solution for hybridization, a buffer of pH 6.5 containing 5

×SSC, 3×Denhard's solution, 0.2% of SDS, 10% of dextran sulfate, 50% of formamide, 0.01% of Antiform B (trade name; manufactured by Sigma) (a defoaming agent), 0.2 mg/ml of DNA modified by salmon sperm, 2.5 mM of sodium pyrophosphate and 25 mM of MES was used. The cDNA moiety integrated in λ Ziplox phage was integrated into plasmid pZL1 for the determination of a base sequence and further subcloned to a plasmid pBluescript IISK (manufactured by Stratagene).

[0041]

cRNA (RNA complementary to cDNA) was prepared from a plasmid containing cDNA of the above-prepared rat dicarboxylate transporter using a T7 RNA polymerase.

[0042]

The resulting cRNA was injected into oocytes of *Xenopus* according to a method by Kanai, et al. (Kanai and Hediger, *Nature*, volume 360, pages 467 to 471, 1992) and, using the oocytes, an uptake experiment was carried out using glutaric acid as a substrate. In the experiment, radioactively labeled substrate (^{14}C-glutaric acid) was used. As a result, uptake of glutaric acid in a sodium-dependent manner was noted and it was confirmed that the cloned cDNA was that of rat dicarboxylate transporter gene (Fig. 1).

[0043]

(2) Cloning of rat kidney organic anion transporter OAT1

It was carried out by an expression cloning method as

follows according to a method by Kanai, et al. (Kanai and Hediger, *Nature*, volume 360, pages 467 to 471, 1992).

[0044]

Rat kidney poly $(A)^+$ RNA (400 μg) was fractionated by a gel electrophoresis.

[0045]

Each fraction obtained by the fractionation was injected into oocytes together with cRNA of the rat dicarboxylate transporter obtained in the above (1). The oocytes used were those which were previously incubated for 2 hours in a sodium uptake solution containing 1 mM of glutaric acid as a substrate (96 mM sodium chloride, 2 mM potassium chloride, 1.8 mM calcium chloride, 1 mM magnesium chloride and 5 mM HEPES; pH 7.4).

[0046]

The oocytes into which RNA was injected were subjected to an experiment for the uptake of substrate using p-aminohippurate (hereinafter, abbreviated as PAH) as a substrate according to a method by Kanai, et al. (Kanai and Hediger, *Nature*, volume 360, pages 467 to 471, 1992). Oocytes were incubated for 1 hour in a sodium uptake solution containing ^{14}C-PAH (50 μM) as a substrate and containing no glutaric acid and the uptake rate of the substrate was measured by way of counting the radioactivity taken up into the cells. It was incidentally confirmed that, in this system, uptake of PAH was not noted in oocytes where only poly$(A)^+$ RNA (mRNA) of kidney

of rat was injected and in oocytes where only cRNA of rat dicarboxylate transporter was injected while, in oocytes where both poly$(A)^+$ RNA of kidney of rat and dicarboxylate transporter cRNA of rat were injected, uptake of PAH was noted (Fig. 2).

[0047]

Among the RNA fractions, there was selected a fraction where the oocytes into which RNA was injected showed the highest uptake rate of PAH. With regard to poly$(A)^+$ RNA (1.8 to 2.4 kb) of this fraction, a cDNA library was prepared using a kit for cDNA synthesis and plasmid cloning (trade name: Superscript Plasmid Systems; manufactured by Gibco). Those DNAs were integrated with the sites recognizing SalI and NotI which were restriction enzymes of plasmid pSPORT1 (manufactured by Gibco) and the resulting recombinant plasmid DNA was introduced into competent cells (trade name: Electro Max DH10B Competent Cell; manufactured by Gibco BRL) of DH10B strain of *Escherichia coli*. The resulting transformant was incubated on a nitrocellulose membrane to give about 500 colonies per plate. Plasmid DNAs were prepared from those colonies and cleaved with a restriction enzyme NotI. Capped cRNA was synthesized using the resulting DNAs by means of an *in vitro* transcription.

[0048]

The resulting cRNA (about 10 ng) was injected into oocytes together with cRNA (2 ng) of the rat dicarboxylate transporter obtained in the above (1). Those oocytes were subjected, as

same as above, to a screening for positive clone by carrying out an uptake experiment for PAH. In the screening, investigation was conducted for a group where DNA extracted from a plurality of clones was pooled and, when uptake of p-aminohippuric acid was confirmed in a group, it was further divided into plural groups followed by conducting a screening.

[0049]

As a result of the screening, one positive clone (clone where uptake of a substrate was noted in oocytes into which cRNA was injected) was isolated from 8,000 clones.

[0050]

With regard to the resulting clone or, in other words, the clone containing cDNA of rat dicarboxylate transporter OAT1, its base sequence was determined by a dideoxy method using a kit for the preparation of deleted clone for determining the base sequence (trade name: Kilo-Sequence Deletion Kit; manufactures by Takara Shuzo), synthetic primer and a kit for the determination of base sequence (trade name: Sequenase ver. 2.0; manufactured by Amersham).

[0051]

As a result, there was obtained a base sequence of cDNA of the rat dicarboxylate transporter OAT1 gene. Further, the base sequence of cDNA was analyzed by a conventional means whereupon the translation region on cDNA and an amino acid sequence of OAT1 coded thereon were determined.

[0052]

Those sequences were shown in SEQ ID No. 1 of the Sequence Listing which will be given later.

[0053]

As a result of analysis of an amino acid sequence of OAT1 by a Kyte-Doolittle hydropathy analysis (hydrophobic plot), 12 membrane-spanning domains were predicted as shown in Fig. 3. In addition, 5 sugar chain-added sites were predicted in the first hydrophilic loop. In a loop of a hydrophilic group of the 6th and 7th transmembrane domains, there were 4 sites which were thought to be proteinkinase C-dependent phosphorylated sites.

[0054]

(3) Expression of OAT1 gene in various tissues (analysis by a northern blotting)

A full-length cDNA of rat OAT1 gene was labeled with ^{32}P-dCTP and, using it as a probe, a northern blotting was carried out as follows to RNA extracted from various tissues of rat. Poly(A)$^+$ RNA (3 μg) was subjected to electrophoresis using 1% agarose/formaldehyde gel and transferred to a nitrocellulose filter. This filter was subjected to hybridization at 42°C for one night using a hybridization solution containing a full-length OAT1 cDNA labeled with ^{32}P-dCTP. The filter was washed at 65°C with 0.1 × SSC containing 0.1% of SDS.

[0055]

As a result of the northern blotting (Fig. 4), in kidney, two bands corresponding to about 2.9 kb and 3.9 kb/4.2 kb were detected whereby expression was noted. In cortex of kidney and outer layer of medulla, expressed amount of OAT1 mRNA was large while it was small in inner layer of medulla.

[0056]

When exposed to light for longer time, a slight band was detected at about 2.4 kb in brain while, in all other tissues, no band was detected and no expression was noted.

[0057]

(4) Expression of OAT1 gene in renal tissues (analysis by an *in situ* hybridization)

An *in situ* hybridization was carried out as follows. Thus, after kidney of rat was fixed by perfusion with 4% paraformaldehyde, it was finely cut and further fixed with 4% paraformaldehyde. The resulting kidney of rat was sliced in a thickness of 5 μm and the resulting slices were used for an *in situ* hybridization.

[0058]

From a full-length OAT1 cDNA were synthesized ^{35}S-labeled sense cRNA and antisense cRNA using T7 or T3 RNA polymerase and they were used as probes. The slice was subjected to hybridization with the probe for one night using a hybridization solution followed by washing with 0.1 × SSC at 37°C for 30 minutes.

[0059]

It was shown as a result of the *in situ* hybridization that, in the layered sites of kidney of rat, OAT1 mRNA was expressed in cortex of kidney and outer layer of medulla, particularly at the part of medullar ray of cortex. In the inner layer of medulla, no expression was detected. The result shows that the organic anion transporter OAT1 was most abundantly expressed in the middle part of proximal convoluted tubule.

[0060]

Example 2. Characterization of organic anion transporter OAT1

(1) Influence of glutaric acid on transport activity of OAT1

In an uptake experiment of PAH by oocytes into which rat OAT1 gene cRNA was injected, influence of preincubation with glutaric acid was investigated.

[0061]

The uptake experiment of PAH was carried out as follows according to a method described in (2) of the above Example 1. Thus, oocytes into which rat OAT1 gene cRNA or rat OAT1 gene cRNA and rat NaDC-1 cRNA was/were injected were subjected to a preincubation for 2 hours in a sodium uptake solution to which 1 mM glutaric acid was or was not added, then ^{14}C-PAH was added, the mixture was incubated at room temperature for 1 hour and uptake of the substrate labeled with radioactivity was measured.

[0062]

As a result, uptake of PAH increased when the oocytes were subjected to a pretreatment with 1 mM glutaric acid (Fig. 5). When oocytes where rat dicarboxylate transporter and OAT1 were expressed were subjected to a pretreatment with glutaric acid, further increase in uptake of ^{14}C-p-aminohippurate was noted. The effect of glutaric acid noted in such a result shows the dependency of PAH uptake on the dicarboxylate concentration in cells whereby it is likely that OAT1 is an exchange transporter for organic anion and dicarboxylic acid.

[0063]

(2) Dependency of transport activity of OAT1 on salt

In an uptake experiment of PAH by oocytes into which rat OAT1 gene cRNA was injected, influence of a salt which was added to a medium was investigated.

[0064]

An uptake experiment for PAH was carried out according to a method mentioned in the above (1) using oocytes into which rat OAT1 gene cRNA was injected. With regard to an uptake solution, however, a choline chloride uptake solution (a sodium solution of 96 mM sodium chloride was changed to 96 mM choline chloride followed by adjusting to pH 7.4) when influence of addition of choline chloride ion was added was checked instead of sodium uptake solution.

[0065]

The result (Fig. 6) was that, even when sodium outside the cells was substituted with choline, no influence was noted on the uptake of PAH. Therefore, it was noted that OAT1 was a transporter which acted in an independent basis to sodium ion.

[0066]

(3) Michaelis-Menten's dynamic test for OAT1

A Michaelis-Menten's dynamic test was carried out for an organic anion transporter. Changes in the uptake rate of PAH by the changes in concentration of the substrate PAH were checked whereupon a Michaelis-Menten's dynamic test for organic anion transporter was carried out.

[0067]

An uptake experiment for PAH was carried out according to the method mentioned in (1) using oocytes into which rat OAT1 gene cRNA was injected. Uptake of ^{14}C-PAH was measured for 3 minutes however. The result (Fig. 7) was that Km value was about 14.3 ± 2.9 μM.

[0068]

The Km value was similar to the Km value (80 μM) for an organic anion transport system at the side of base reported in an *in vivo* system already (Ulrich, et al., *Am. J. Physiol.*, volume 254, pages F453 to 462, 1988).

[0069]

(4) Substrate selectivity of OAT1 (Inhibiting test by

addition of drug)

In a PAH uptake experiment using oocytes into which rat OAT1 gene cRNA was injected, influence of addition of various drugs to the system was checked.

[0070]

The PAH uptake experiment was carried out according to the method mentioned in the above (1) using oocytes into which rat OAT1 gene cRNA was injected. However, a sodium uptake solution was used and uptake of PAH was measured in the presence and absence of various compounds (non-labeled) in an amount of 2 mM.

[0071]

The result (Fig. 8) was that a cis-inhibiting effect was observed by addition of a drug which was unrelated to structure. Cefaloridin (antibiotic substance of a β-lactam type), nalidixic acid (old quinolone), furosemide and ethacrynic acid (diuretics), indomethacin (nonsteroidal anti-inflammatory agent), probenecid (agent for excretion of uric acid) and valproic acid (anti-epilepsy agent) strongly inhibited the uptake of ^{14}C-p-aminohippurate mediated by OAT-1 (85%>). Methotrexate which is an anti-tumor agent inhibited the uptake of PAH in a medium degree. Endogenous compounds such as prostaglandin E2, c-AMP, c-GMP and uric acid also inhibited the uptake of PAH.

[0072]

(5) Substrate-specificity of OAT1 (Uptake test where various kinds of anionic substances were used as substrates)

Uptake by OAT1 was investigated using various kinds of anionic substances as substrates.

[0073]

The PAH uptake experiment was carried out according to the method mentioned in the above (1) using oocytes into which rat OAT1 gene cRNA was injected. As to the substrate however, various compounds labeled with radioactivity were used in place of ^{14}C-PAH.

[0074]

The result (Fig. 9) was that uptake to oocytes was noted when methoxalate (^{3}H-labeled substance), c-AMP (^{3}H-labeled substance), c-GMP (^{3}H-labeled substance), prostaglandin (^{3}H-labeled substance), uric acid (^{14}C-labeled substance) and α-ketoglutaric acid (^{14}C-labeled substance) were used as substrates. On the other hand, no uptake was noted in the case of TEA (^{14}C-labeled substance) and taurocholic acid.

[0075]

Example 3. Cloning of human organic anion transporter

The cDNA fragment of rat OAT1 gene obtained in (2) of Example 1 was labeled and was used as a probe for the screening of human cDNA library. With regard to the human cDNA library, there was used a human cDNA library prepared by using human kidney poly(A)$^{+}$ RNA (manufactured by Clontec) as a gene source.

[0076]

With regard to the resulting positive clone, or a clone containing human organic anion transporter (human OAT1) cDNA, its base sequence was determined by the same manner as in Example 1 and the base sequence of the resulting cDNA was analyzed by a conventional method to determine the translation region on cDNA and the amino acid sequence of human OAT1 coded thereon.

[0077]

Sequence of the human OAT1 is shown in SEQ ID No. 2 in the Sequence Listing which will be mentioned later.

[0078]

Homology of rat OAT1 to human OAT1 was about 85% at an amino acid level. Homology at a cDNA level was about 79%.

[0079]

[Advantage of the Invention]

The organic anion transporter OAT1 of the present invention and gene thereof is expected to be useful for clarification of the fate of a drug and the fate of a toxin at a molecular level such as an *in vitro* analysis of excretion of a drug and interaction between drugs. Further, many drugs which are causes for renal failure such as antibiotic substance of β lactam type, diuretics and nonsteroidal anti-inflammatory agents are transported by OAT1 and the cause of induction of renal toxicity by drugs is suggested to be possibly by accumulation due to OAT1 whereby a method for screening a drug

for prevention of renal toxicity using OAT1 is expected to be developed.

[0080]

[SEQUENCE LISTING]

Sequence ID No: 1

Length: 2294

Type: Nucleic acid

Number of Chain : Doubled-Stranded

Topology : Linear

Kind : cDNA to mRNA

Origin

Organism: Rat

Sequence

```
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CCTGCCACAG AACCGGCTCA GCTCCAGCTC CAGGAGTCAC TCAGCTGCAG AGGCAGTGGC  180
AGCCCCACTC CTCAGGCAAA GGGCAGCAGA CAGACAGACA GAGGTCCTAG GACTGGAGGT  240
CCTCAGTCAT TGACCACTCA GCCTGGCCCA GCCCC                            275
ATG GCC TTC AAT GAC CTC CTG AAA CAG GTG GGG GGC GTC GGA CGC        320
Met Ala Phe Asn Asp Leu Leu Lys Gln Val Gly Gly Val Gly Arg
 1               5                  10                  15
TTC CAG TTG ATC CAG GTC ACC ATG GTG GTT GCT CCC CTA CTG CTG        365
Phe Gln Leu Ile Gln Val Thr Met Val Val Ala Pro Leu Leu Leu
                20                  25                  30
ATG GCT TCC CAC AAC ACC TTG CAG AAC TTC ACT GCC GCT ATC CCC        410
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Met Ala Ser His Asn Thr Leu Gln Asn Phe Thr Ala Ala Ile Pro

35 40 45

CCT CAT CAC TGC CGC CCA CCT GCC AAT GCC AAT CTC AGC AAA GAT 455

Pro His His Cys Arg Pro Pro Ala Asn Ala Asn Leu Ser Lys Asp

50 55 60

GGA GGT CTG GAG GCC TGG CTG CCC CTG GAC AAG CAA GGA CAA CCC 500

Gly Gly Leu Glu Ala Trp Leu Pro Leu Asp Lys Gln Gly Gln Pro

65 70 75

GAA TCG TGC CTC CGC TTT ACT TCC CCC CAG TGG GGA CCA CCC TTT 545

Glu Ser Cys Leu Arg Phe Thr Ser Pro Gln Trp Gly Pro Pro Phe

80 85 90

TAC AAT GGC ACA GAA GCC AAT GGC ACC AGA GTC ACA GAG CCC TGC 590

Tyr Asn Gly Thr Glu Ala Asn Gly Thr Arg Val Thr Glu Pro Cys

95 100 105

ATT GAT GGC TGG GTC TAT GAC AAC AGC ACC TTC CCT TCA ACC ATC 635

Ile Asp Gly Trp Val Tyr Asp Asn Ser Thr Phe Pro Ser Thr Ile

110 115 120

GTG ACT GAG TGG AAC CTT GTG TGC TCT CAT CGG GCT TTC CGC CAG 680

Val Thr Glu Trp Asn Leu Val Cys Ser His Arg Ala Phe Arg Gln

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Leu Ala Gln Ser Leu Tyr Met Val Gly Val Leu Leu Gly Ala Met

140 145 150

GTG TTT GGC TAC CTG GCG GAC AGG CTG GGC CGC CGG AAG GTG CTG 770

Val Phe Gly Tyr Leu Ala Asp Arg Leu Gly Arg Arg Lys Val Leu

155 160 165

ATC TTG AAC TAC CTG CAG ACA GCT GTG TCG GGA ACC TGT GCA GCC 815

Ile Leu Asn Tyr Leu Gln Thr Ala Val Ser Gly Thr Cys Ala Ala

170 175 180

TAT GCA CCC AAC TAT ACT GTC TAC TGC GTT TTC CGG CTC CTC TCG 860

Tyr Ala Pro Asn Tyr Thr Val Tyr Cys Val Phe Arg Leu Leu Ser

185 190 195

GGC ATG TCT TTG GCT AGC ATT GCA ATC AAC TGC ATG ACA CTA AAT 905

Gly Met Ser Leu Ala Ser Ile Ala Ile Asn Cys Met Thr Leu Asn

200 205 210

GTG GAA TGG ATG CCT ATC CAC ACC CGT GCC TAT GTG GGC ACC TTG 950

Val Glu Trp Met Pro Ile His Thr Arg Ala Tyr Val Gly Thr Leu

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230 235 240

GCC TAT GCT GTG CCC CAC TGG CGC CAC CTG CAG CTT GTG GTC TCT 1040

Ala Tyr Ala Val Pro His Trp Arg His Leu Gln Leu Val Val Ser

245 250 255

GTG CCT TTT TTC ATT GCC TTC ATC TAC TCT TGG TTC TTC ATT GAG 1085

Val Pro Phe Phe Ile Ala Phe Ile Tyr Ser Trp Phe Phe Ile Glu

260 265 270

TCA GCC CGC TGG TAC TCC TCC TCA GGA AGG CTG GAC CTC ACC CTC 1130

Ser Ala Arg Trp Tyr Ser Ser Ser Gly Arg Leu Asp Leu Thr Leu

275 280 285

CGA GCC CTG CAG AGA GTG GCC CGG ATC AAT GGG AAA CAA GAA GAA 1175

Arg Ala Leu Gln Arg Val Ala Arg Ile Asn Gly Lys Gln Glu Glu

290 295 300

GGG GCT AAG CTA AGT ATA GAG GTG CTC CGG ACC AGC CTG CAG AAG 1220

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320 325 330

CGC TGC CCC ACC CTT CGA CAC CTC TTC CTC TGT CTC TCC ATG CTG 1310

Arg Cys Pro Thr Leu Arg His Leu Phe Leu Cys Leu Ser Met Leu

335 340 345

TGG TTT GCC ACT AGC TTT GCC TAC TAC GGG CTG GTC ATG GAC CTG 1355

Trp Phe Ala Thr Ser Phe Ala Tyr Tyr Gly Leu Val Met Asp Leu

350 355 360

CAG GGC TTT GGG GTC AGC ATG TAC CTT ATC CAG GTG ATT TTC GGT 1400

Gln Gly Phe Gly Val Ser Met Tyr Leu Ile Gln Val Ile Phe Gly

365 370 375

GCC GTG GAC CTG CCT GCC AAG TTT GTA TGC TTC CTA GTC ATC AAC 1445

Ala Val Asp Leu Pro Ala Lys Phe Val Cys Phe Leu Val Ile Asn

380 385 390

TCC ATG GGG CGC CGG CCT GCA CAG ATG GCC TCC CTG CTG CTG GCA 1490

Ser Met Gly Arg Arg Pro Ala Gln Met Ala Ser Leu Leu Leu Ala

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Gly Ile Cys Ile Leu Val Asn Gly Ile Ile Pro Lys Ser His Thr

410 415 420

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Ile Ile Arg Thr Ser Leu Ala Val Leu Gly Lys Gly Cys Leu Ala

425 430 435

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Ser Ser Phe Asn Cys Ile Phe Leu Tyr Thr Gly Glu Leu Tyr Pro

440 445 450

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Thr Val Ile Arg Gln Thr Gly Leu Gly Met Gly Ser Thr Met Ala

455 460 465

CGG GTG GGC AGC ATT GTG AGC CCG CTG GTG AGC ATG ACT GCA GAG 1715

Arg Val Gly Ser Ile Val Ser Pro Leu Val Ser Met Thr Ala Glu

470 475 480

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Phe Tyr Pro Ser Met Pro Leu Phe Ile Phe Gly Ala Val Pro Val

485 490 495

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Val Ala Ser Ala Val Thr Ala Leu Leu Pro Glu Thr Leu Gly Gln

500 505 510

CCG CTG CCA GAT ACA GTG CAG GAC CTG AAG AGC AGG AGC AGA GGA 1850

Pro Leu Pro Asp Thr Val Gln Asp Leu Lys Ser Arg Ser Arg Gly

515 520 525

AAG CAG AAT CAA CAG CAG CAG GAA CAG CAG AAG CAG ATG ATG CCG 1895

Lys Gln Asn Gln Gln Gln Gln Glu Gln Gln Lys Gln Met Met Pro

530 535 540

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Leu Gln Ala Ser Thr Gln Glu Lys Asn Gly Leu

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AAAAAA 2294

[0081]

Sequence ID No: 2

Length: 2171

Type: Nucleic acid

Number of Chain : Doubled-Stranded

Topology : Linear

Kind : cDNA to mRNA

Origin

Organism: Human

Sequence

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GCAGCTCGGA CTCAGCTCCC GGAGCAACCC AGCTGCGGAG GCAACGGCAG TGCTGCTCCT 180

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GAG TCC TGC CTC CGC TTC ACC TCC CCG CAG TGG GGA CTG CCC TTT 537

Glu Ser Cys Leu Arg Phe Thr Ser Pro Gln Trp Gly Leu Pro Phe

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Ala Tyr Ala Val Pro His Trp Arg His Leu Gln Leu Leu Val Ser

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260 265 270

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290 295 300

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Glu Leu Thr Met Gly Lys Gly Gln Ala Ser Ala Met Glu Leu Leu

320 325 330

CGC TGC CCC ACC CTC CGC CAC CTC TTC CTC TGC CTC TCC ATG CTG 1302

Arg Cys Pro Thr Leu Arg His Leu Phe Leu Cys Leu Ser Met Leu

335 340 345

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365 370 375

GCT GTG GAC CTG CCT GCC AAG CTT GTG GGC TTC CTT GTC ATC AAC 1437

Ala Val Asp Leu Pro Ala Lys Leu Val Gly Phe Leu Val Ile Asn

380 385 390

TCC CTG GGT CGC CGG CCT GCC CAG ATG GCT GCA CTG CTG CTG GCA 1482

Ser Leu Gly Arg Arg Pro Ala Gln Met Ala Ala Leu Leu Leu Ala

395 400 405

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Thr Met Ile Arg Gln Thr Gly Met Gly Met Gly Ser Thr Met Ala

455 460 465

CGA GTG GGC AGC ATC GTG AGC CCA CTG GTG AGC ATG ACT GCC GAG 1707

Arg Val Gly Ser Ile Val Ser Pro Leu Val Ser Met Thr Ala Glu

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500 505 510

CCA CTG CCA GAC ACG GTG CAG GAC CTG GAG AGC AGG TGG GCC CCC 1842

Pro Leu Pro Asp Thr Val Gln Asp Leu Glu Ser Arg Trp Ala Pro

515 520 525

ACT CAG AAA GAA GCA GGG ATA TAT CCC AGG AAA GGG AAA CAG ACG 1887

Thr Gln Lys Glu Ala Gly Ile Tyr Pro Arg Lys Gly Lys Gln Thr

530 535 540

CGA CAG CAA CAA GAG CAC CAG AAG TAT ATG GTC CCA CTG CAG GCC 1932

Arg Gln Gln Gln Glu His Gln Lys Tyr Met Val Pro Leu Gln Ala

545 550 555

TCA GCA CAA GAG AAG AAT GGA CTC 1956

Ser Ala Gln Glu Lys Asn Gly Leu

560 563

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GGAAAGCATC TTCCCAGGGG TCCACCTCCC TTTATAAACC CCACCAGAAC CACATCATTA 2136

AAAGGTTTGA CTGCGAAAAA AAAAAAAAAA AAAAA 2171

[Brief Description of the Drawings]

[Fig. 1] This is a drawing which shows the result of an uptake experiment of glutaric acid by oocytes into which cRNA of sodium-dependent dicarboxylate transporter (NaDC-1) gene of rat was injected.

[Fig. 2] This is a drawing which shows the result of an uptake experiment of PAH by oocytes into which mRNA derived from renal tissues of rat and/or cRNA of NaDC-1 gene of rat were/was injected.

[Fig. 3] This is a drawing which shows hydrophobic plots of rat organic anion transporter OAT1.

[Fig. 4] This is a photographic picture which shows the result where expression of OAT1 gene mRNA in various organ tissues of rat was analyzed by a northern blotting.

[Fig. 5] This is a drawing which shows the result where influence of preincubation with glutaric acid was investigated in a PAH uptake experiment by oocytes into which rat OAT1 gene cRNA was injected.

[Fig. 6] This is a drawing which shows the result of investigation of influence of salt added in a PAH uptake experiment by oocytes into which rat OAT1 gene cRNA was injected.

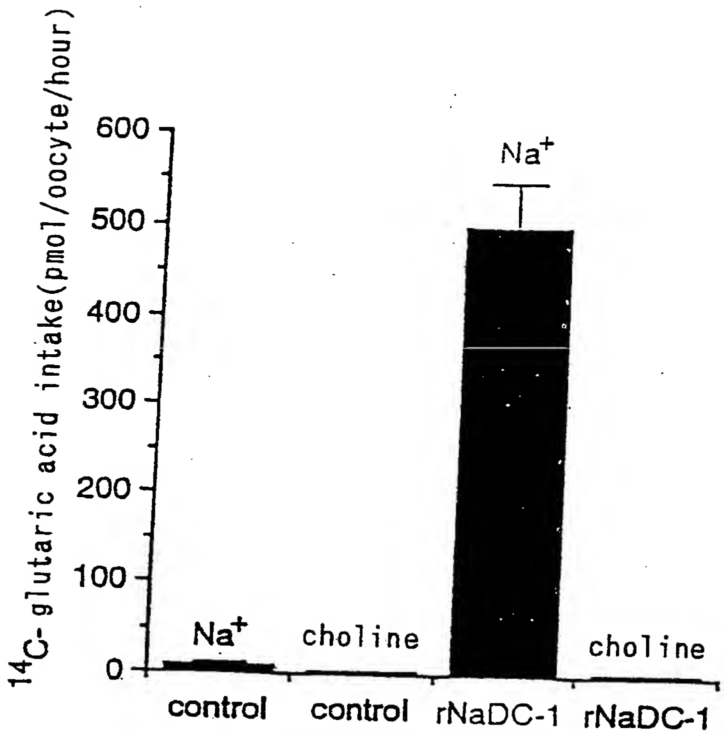
[Fig. 7] This is a drawing which shows the result of investigation of influence of concentration of the substrate PAH in a PAH uptake experiment by oocytes into which rat OAT1 gene cRNA was injected.

[Fig. 8] This is a drawing which shows the result of investigation of influence of addition of various drugs to the system in a PAH uptake experiment by oocytes into which rat OAT1 gene cRNA was injected.

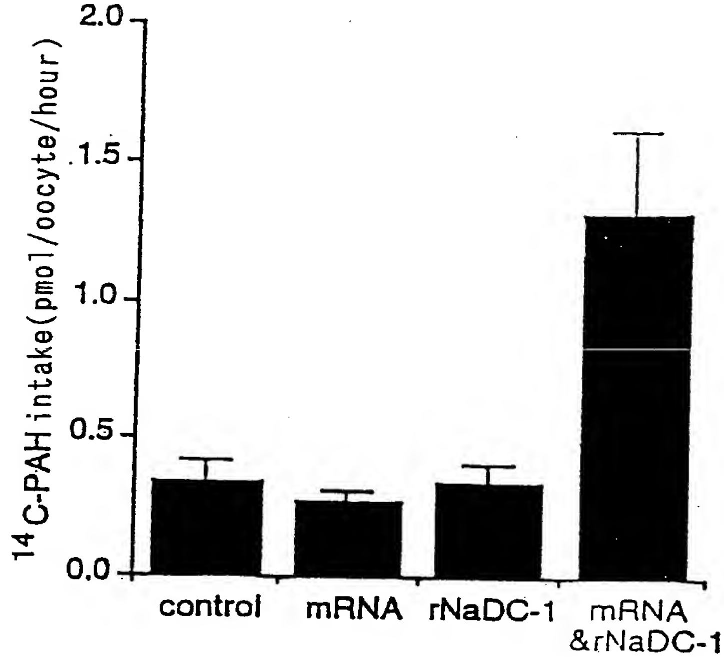
[Fig. 9] This is a drawing which shows the result of an uptake experiment by oocytes into which cRNA of rat OAT1 gene was injected when various drugs were used as substrates.

【Document Name】 Drawings

【Figure 1】

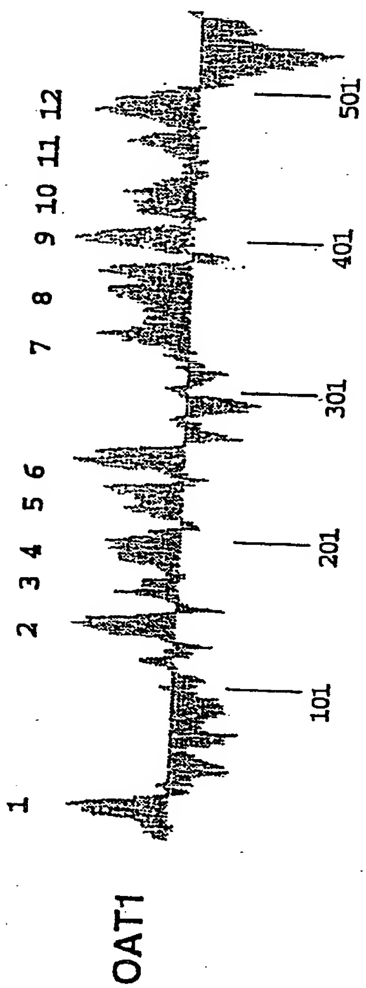


【Figure 2】

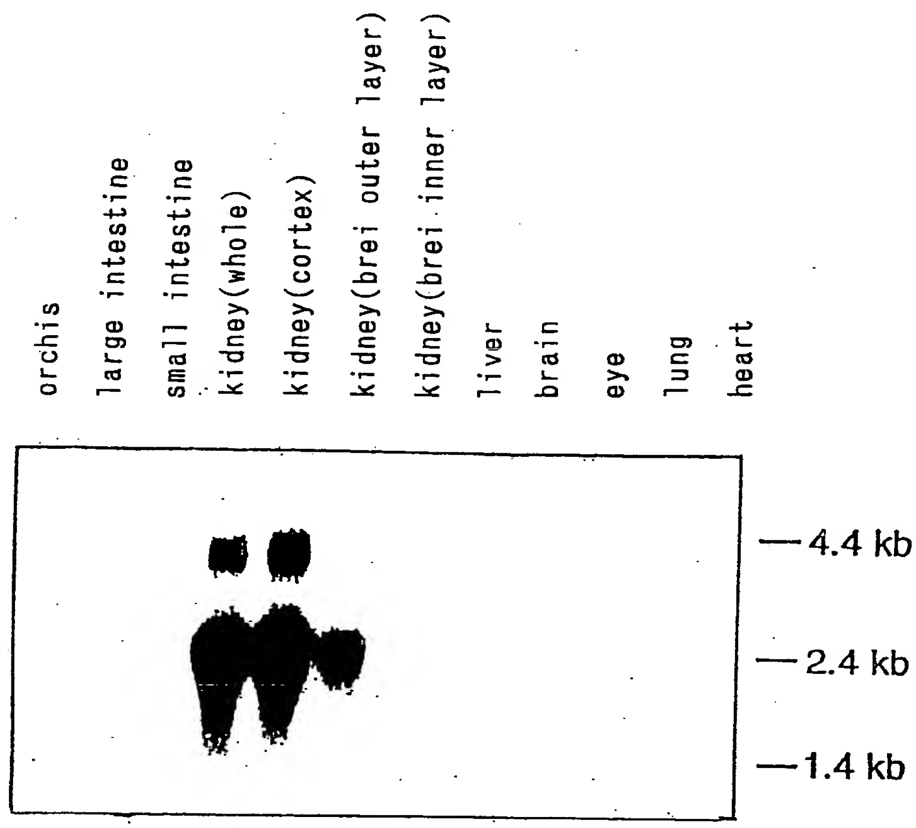


2.0
1.5
1.0
0.5
0.0
14C-PAH intake(pmol/oocyte/hour)
control
mRNA
rNaDC-1
mRNA
&rNaDC-1

【Figure 3】

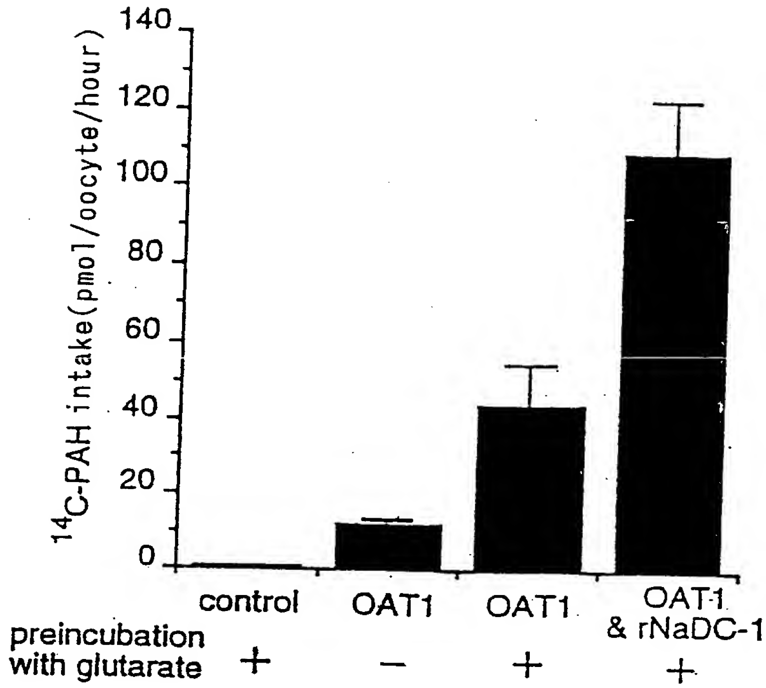


【Figure 4】



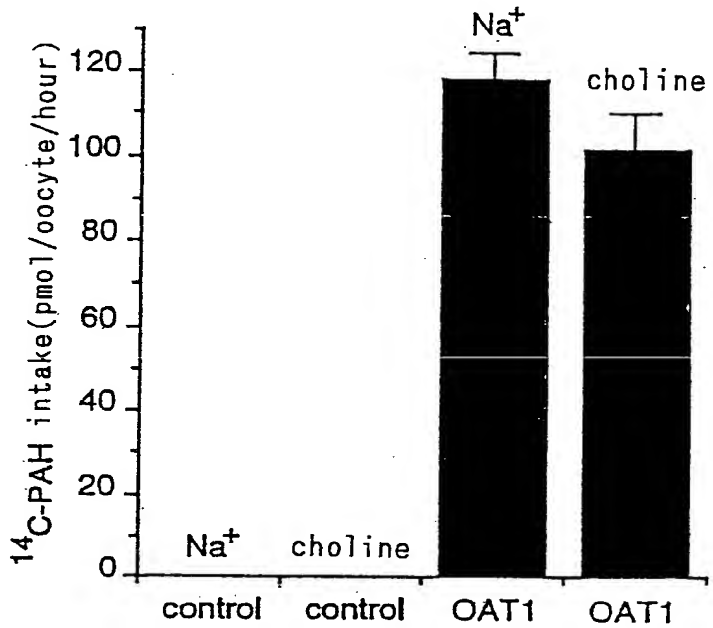
orchis
large intestine
small intestine
kidney(whole)
kidney(cortex)
kidney(brei outer layer)
kidney(brei inner layer)
liver
brain
eye
lung
heart
4.4 kb
2.4 kb
1.4 kb

[Figure 5]



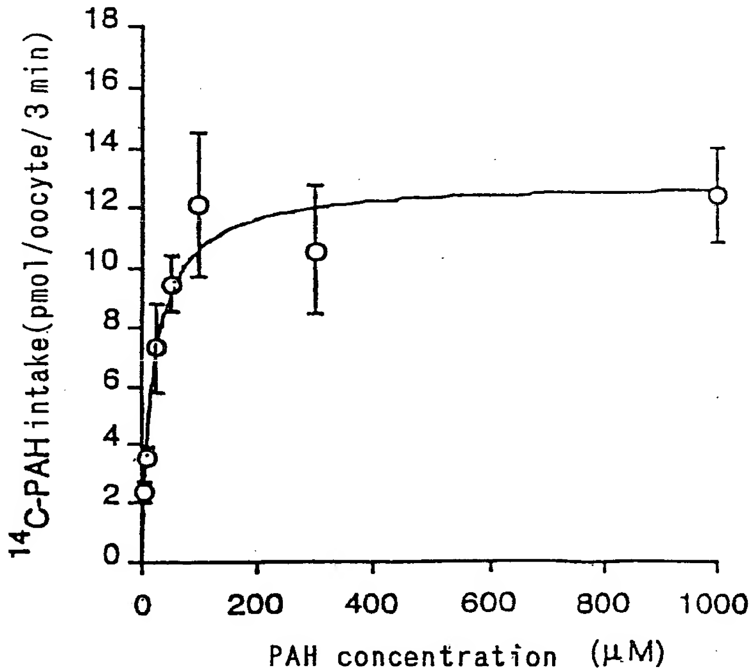
140
120
100
80
60
40
20
0
^{14}C-PAH intake (pmol/oocyte/hour)
control
OAT1
OAT1
OAT-1
& rNaDC-1
preincubation
with glutarate
+
−
+
+

【Figure 6】



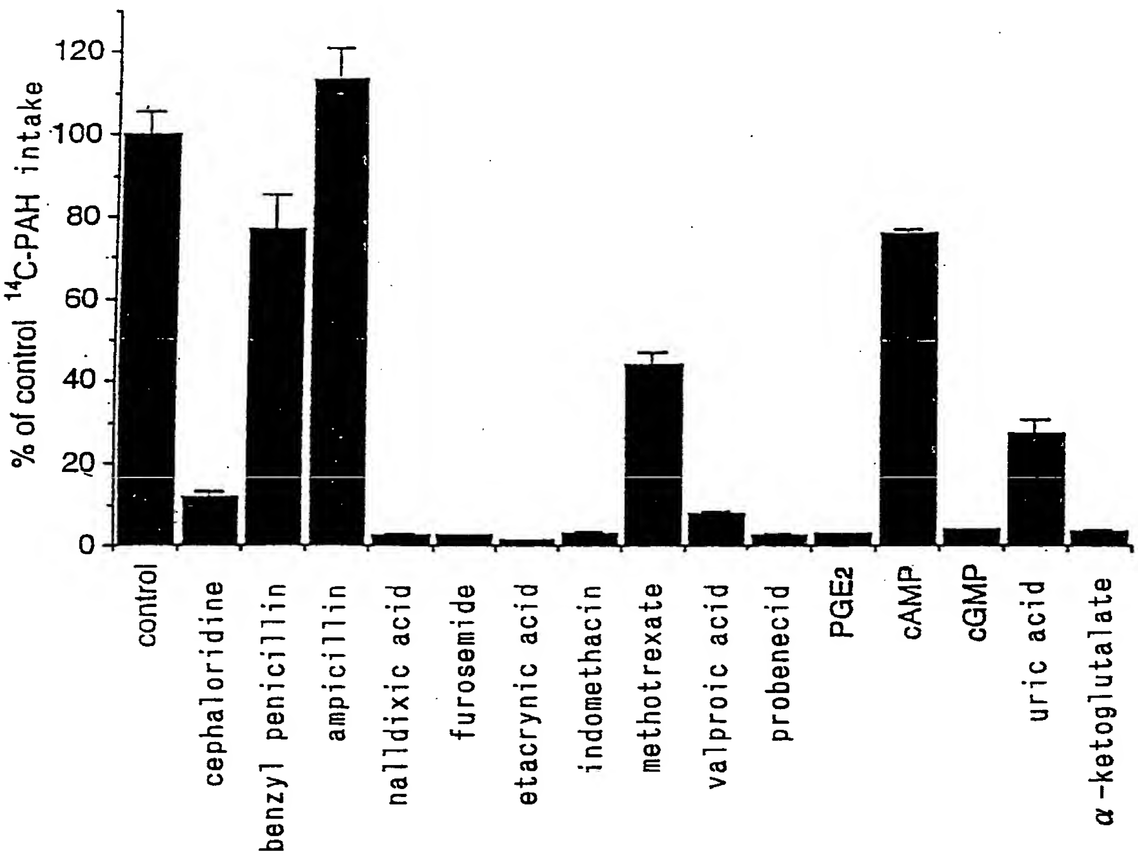
^{14}C-PAH intake (pmol/oocyte/hour)
120
100
80
60
40
20
0
Na+
choline
Na+
choline
control
control
OAT1
OAT1

【Figure 7】

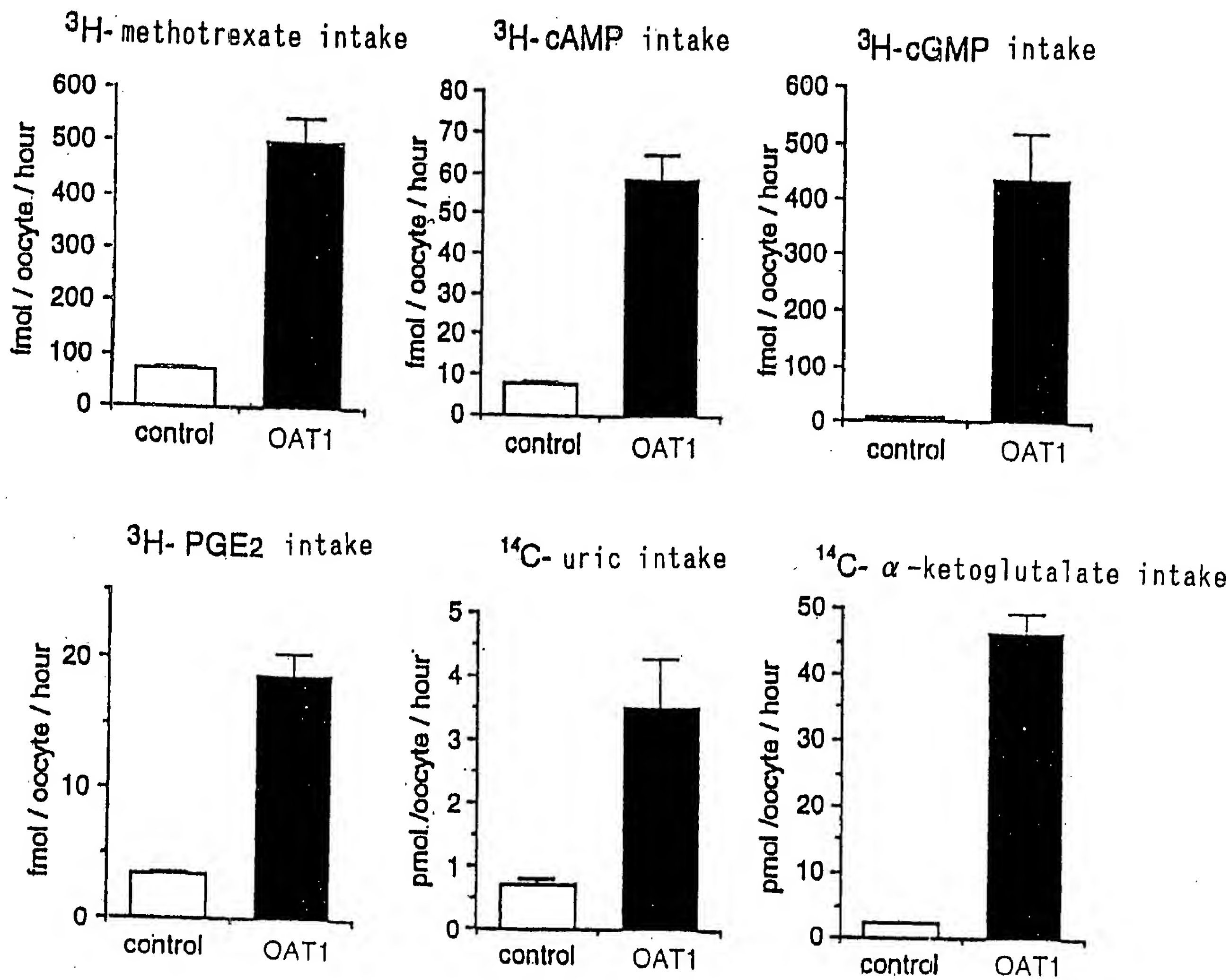


^{14}C-PAH intake (pmol/oocyte/3 min)
0
2
4
6
8
10
12
14
16
18
0
200
400
600
800
1000
PAH concentration (μM)

[Figure 8]


% of control ¹⁴C-PAH intake
120
100
80
60
40
20
0
control
cephaloridine
benzyl penicillin
ampicillin
nalldixic acid
furosemide
etacrynic acid
indomethacin
methotrexate
valproic acid
probenecid
PGE2
cAMP
cGMP
uric acid
α-ketoglutalate

[Figure 9]



³H- methotrexate intake
fmol / oocyte / hour
600
500
400
300
200
100
0
control
OAT1
³H-cAMP intake
fmol / oocyte / hour
80
70
60
50
40
30
20
10
0
control
OAT1
³H-cGMP intake
fmol / oocyte / hour
600
500
400
300
200
100
0
control
OAT1
³H- PGE2 intake
fmol / oocyte / hour
20
10
0
control
OAT1
¹⁴C- uric intake
pmol /oocyte / hour
5
4
3
2
1
0
control
OAT1
¹⁴C- α-ketoglutalate intake
pmol /oocyte / hour
50
40
30
20
10
0
control
OAT1

[Designation of Document] Abstract

[Abstract]

[Problem] Novel organic anion transporter and gene coding thereof are provided.

[Means for Resolution] Protein having a capability of transporting an organic anion and comprising an amino acid sequence represented by SEQ ID No. 1 or 2 or an amino acid sequence represented by SEQ ID No. 1 or 2 where one or several amino acid(s) is/are deleted therefrom, substituted therefor or added thereto. Gene coding the above-mentioned protein.

[Selected Drawing] (none)